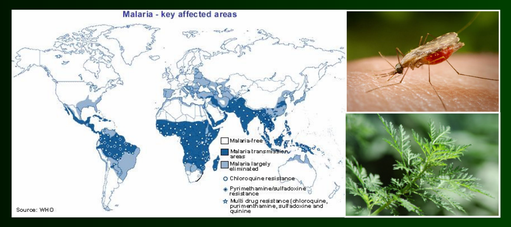


High-cell density fermentation process for the production of anti-malarial therapy precursor, Artemisinic Acid in *E. coli*

Tizita Horning[1], Douglas J. Pitera[1], Bonny Lieu[1], Michelle C. Chang[2], Jay D. Keasling[2], Jack D. Newman[1], and Rika Regentin[1].
[1] Amyris Biotechnologies, Inc. 5980 Horton Street, Ste. 450, Emeryville, CA 94608
[2] California Institute for Quantitative Biomedical Research, University of California, Berkeley, CA 94720-3224

Introduction

Malaria is a disease common to tropical and subtropical regions throughout the world that kills more than one million people per year, mostly children. Malarial infection is caused by strains of *Plasmodium falciparum* and related species, many of which have become resistant to prior, low-cost, chloroquine drug based treatments, greatly hampering control of the disease. The sesquiterpenoid artemisinin is an anti-malarial agent that is highly effective against drug resistant *Plasmodium* strains and is a key component of Artemisinin Based Combination Therapies (ACTs) now endorsed as the frontline therapy to treat the disease[1]; however, artemisinin is seasonal, of variable quality, and can be in short supply, making ACTs unaffordable or unavailable to those most affected by the disease. Synthesis of artemisinin from microbially derived artemisinic acid (direct precursor of artemisinin) is an attractive low-cost alternative to extraction from plant material or total chemical synthesis.

Project Goal

Optimize fed-batch fermentation conditions to achieve high titers of artemisinic acid in engineered *E. coli.*

Materials and Method

Strains

- *E. coli* strains B445 (3-plasmid system) and B569 (2-plasmid system) contain:
  - *S. cerevisiae* mevalonate pathway for production of farnesyl pyrophosphate (FPP)[2]
  - *E. coli* codon optimized amorphadiene synthase (ADS) from *A. annua*[2]
  - Modified amorphadiene oxidase (AMO), a cytochrome P450, and cytochrome P450 reductase (CPR) from *A. annua*[3]

Fermentation Strategy

- Control process adapted from Korz *et al.*[4]
  - Defined medium, glucose limited fed-batch fermentation
  - Exponentially increasing feed rate (6 hr doubling time) after batch phase followed by linear feed rate during stationary growth
  - pH 7, Control process temperature: 30°C
- Heterologous artemisinic acid pathway induced with IPTG at OD_{600} ~30
- Media supplemented with 5-aminoluvonic acid (ALA) at induction to increase intracellular heme concentration (cofactor of cytochrome P450)[3]

Process Development Strain B445

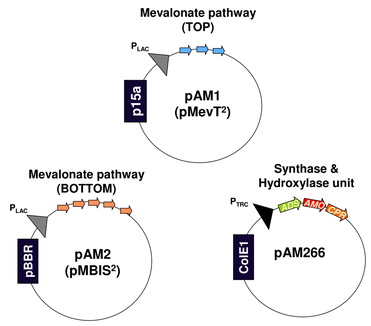
Initial fermentations were conducted using the defined control fermentation parameters. Although strain grew well, very low levels of oxidized sesquiterpene product was observed and none of the target product, artemisinic acid, was detected (Fig. 1).

To improve upon the initial fermentation run, temperature was tested as a variable of study. Reduction of temperature from 30 C to 25 C caused no difference in growth but an overall improvement in sesquiterpene production was observed (Fig. 2).

Amorphadiene feeding studies indicated possible substrate inhibition of AMO (data not shown). By increasing the exponential phase doubling time (Td) from six to eight hours we observed a drop in concentration of intermediate pathway products and detected final product, artemisinic acid, for the first time (Fig. 3).

Combining the best two process conditions (Td = 8 hr and temperature = 25C) did not improve artemisinic acid production but all oxidized products of amorphadiene were detected (Fig. 4).

Engineered *E. coli* Strain B445



Control Process

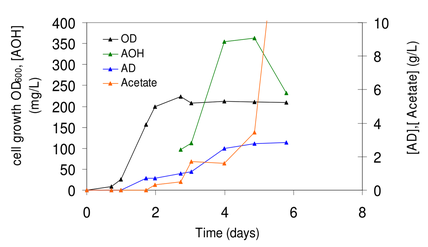


Figure 1

Lower Temperature, 25C

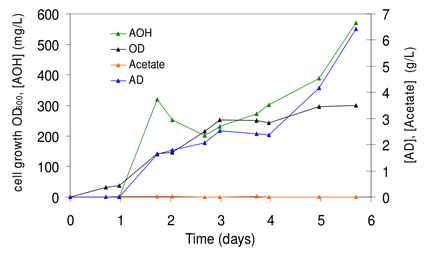


Figure 2

Slower Doubling Time, 30C

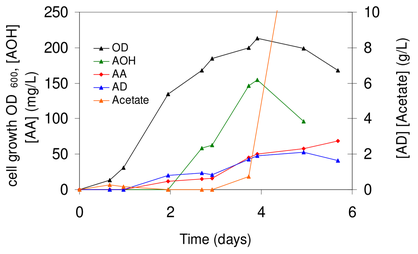


Figure 3

Slow doubling time at low Temperature

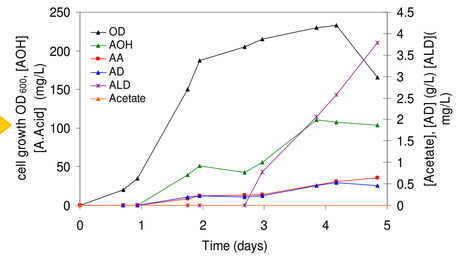
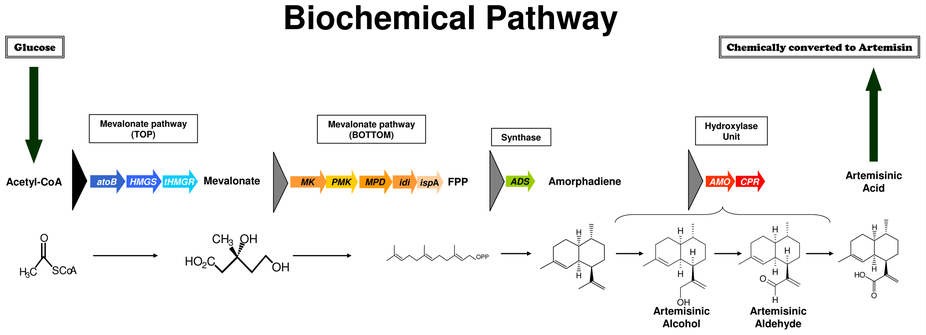


Figure 4

Biochemical Pathway



Process Development Strain B569

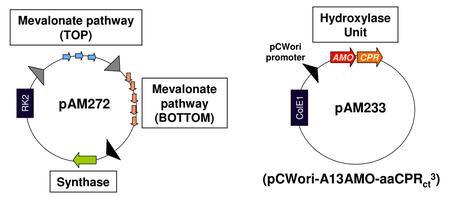
Transferred process parameters that showed improved yields of oxidized products in *E. coli* B445 to initial fermentations of *E. coli* B569. Fermentation of B569 at 25C using 10 hr exponential phase doubling time resulted in growth inhibition, overfeeding, and cell death soon after induction (Fig. 5).

To determine if growth inhibition was due to induction of the pathway enzymes, or overfeeding at low temperature, induction of cells at 25C was delayed until after the end of the exponential feed. Induction during low stationary feed rate did not cause growth inhibition; however, production of artemisinic acid was poor (Fig. 6). Concurrently, we tested reducing the maximum exponential feed rate to account for growth inhibition after induction - culture was still overfed and cell death was observed.

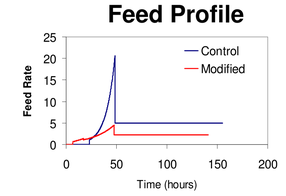
Temperature was further reduced to 20C based on flask experiments which reflected higher titers of artemisinic acid at lower temperature. Fermentation at 20C resulted in an increased rate of artemisinic acid production; however, growth inhibition, acetate accumulation and rapid cell death was observed post-induction (Fig. 7).

By growing B569 at 20C and further modifying feed rate (reduced Td to 15 hr after reduction of temperature), we obtained 1.5 g/L titer of artemisinic acid after induction and observed no growth inhibition (Fig. 8).

Engineered *E. coli* Strain B569



Feed Profile



B569 in 25C, 10 hr doubling time process

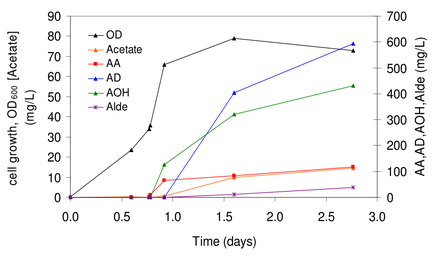


Figure 5

Induction at high optical density, 25C, 6 hour doubling time

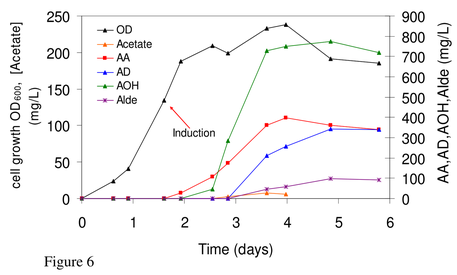


Figure 6

Further Reduction in Temperature to 20C, 12 hour doubling time

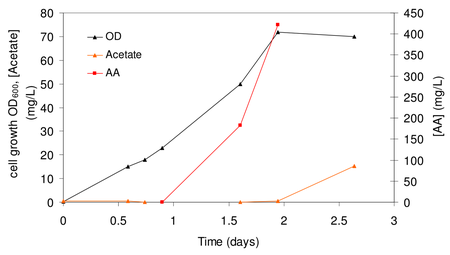


Figure 7

20C, 15 hr doubling time post-temperature change, lower max feed rate

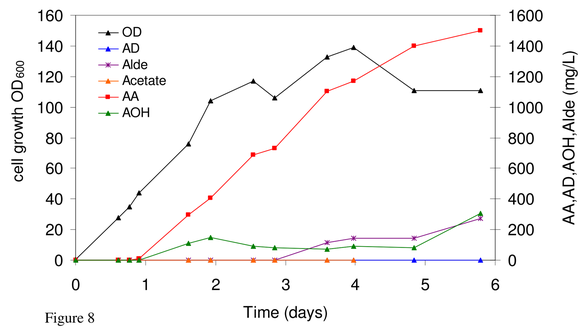


Figure 8

Reducing Max Feed Rate

Reducing the max exponential feed rate did not alleviate growth inhibition. After induction, cells growth ceased, excess carbon was converted into acetate and cell death ensued.

Conclusions

- Reduced feed rate and lower temperature increased production of oxidized products of amorphadiene in *E. coli* B445.
- Amorphadiene oxidase activity in *E.coli* B445 was insufficient for the rate of amorphadiene production. Production and consumption of amorphadine appeared more balanced in *E. coli* B569 under optimized conditions.
- Expression of AMO and CPR from pCWori, in *E. coli* B569, resulted in growth inhibition, overfeeding, acetate accumulation, and cell death.
- Growth inhibition was alleviated and artemisinic acid production increased in *E. coli* B569 by reducing maximum feed rate, increasing exponential phase doubling time, and reducing process temperature.
- Combined strain and process improvements resulted in production of 1.5 g/L artemisinic acid in *E. coli.*

Literature cited

1. Guidelines for the treatment of malaria. World Health Organization. Geneva 2006
2. Martin, V.JJ., Pitera, D.J., Withers, S.T., Newman, J.D., and Keasling, J.D. Engineering Mevalonate Pathway in *Escherichia coli* for the production of terpenoids. *Nature Biotechnology* 21(7): 796-802. (2003)
3. Chang, M.C.Y., Eachus, R.A., Trieu, W., Ro, DK., and Keasling, J.D. Engineering *Escherichia coli* for production of functionalized terpenoids using plant P450s. *Nature chemical biology* 3(5): 274-277 (2007)
4. Korz, D.J., Rinas, U., Hellmuth, K., Sanders, E.A., Deckwer, W.-D. Simple fed-batch technique for high cell density cultivation of *Escherichia coli*. *Journal of Biotechnology* 39: 59-65. (1995)

Acknowledgments

This research was conducted under the sponsorship of the Institute for One World Health through the generous support of The Bill and Melinda Gates Foundation.